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<p>(54) Title: 7-DEAZAPURINE MODIFIED OLIGONUCLEOTIDES</p> <p>(57) Abstract</p> <p>Oligonucleotides, which incorporate 7-deazanucleosides, are useful as antisense sequences to inhibit the function of RNA and DNA.</p>		

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7-DEAZAPURINE MODIFIED OLIGONUCLEOTIDESField of the Invention

5 This invention relates to modified
oligonucleotide sequences containing 7-deazapurine
nucleosides, to a method of inhibiting nuclease degradation
of oligonucleotides incorporating the same, to a method of
10 inhibiting gene expression in a cellular system and to
compositions useful for inhibiting gene expression
containing the modified oligonucleotides.

Information Disclosure Statement

15 Seela and Kehne, Biochem., 26, 2232-2238 (1987)
disclose 7-deazadeoxyadenosine (9- β -2'-deoxyribofuranosyl-
7-deazaadenine) and the incorporation of from one to two
such nucleosides into octa and dodecanucleotides having the
20 palindromic EcoRI endonuclease DNA recognition sequence
d(GAATTC). The oligonucleotides were prepared for study of
their stability to cleavage by EcoRI.

 Seela and Driller, Nucl. Acid. Res., 17(3), 901-
910 (1989) describe the preparation of hexanucleotide
25 sequences containing d(GC)₃ and d(CG)₃ nucleotide units and
such hexamers containing 7-deazaguanosine (c⁷G_d) and 7-
deaza-8-azaguanosine (c⁷z⁸G_d) nucleoside units. The self-
complementary hexamers so-prepared form duplexes which were
prepared for the purpose of studying the stability of the
30 duplexes and the thermodynamic parameters of helix-coil
transition for each of the G-C/C-G base pairs.

 Tran-Thi et al., Angew. Chem. Int. Ed. Engl.,
21(5), 367-368, (1982) disclose the preparation of 7-deaza-
guanosine and the preparation therefrom of cyclic guanosine
35 monophosphate.

 Seela and Kehne, Biochem., 24(26), 7556-7561
(1985) describe the synthesis of self-complementary
hexamers and dodecamers employing solid phase techniques

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and an appropriately protected phosphoramidite for study of
40 the base pairing and base stacking properties as reflected
by their melting curves and their behavior toward snake
venom phosphodiesterase and single strand specific nuclease
SI.

Seela and Driller, Nucl. Acid. Res., 13(3), 911-
45 926 (1985) describe the synthesis of a 3'-phosphoramidite
of 7-deaza-2'-deoxyguanosine and the synthesis of the self-
complementary hexamer of d(CG), where the guanosine
moieties were replaced by 7-deaza-2'-deoxyguanosine, and
study of the properties of the resulting duplex.

50 Winkeler and Seela, J. Org. Chem., 48, 3119-3122
(1983) report the total synthesis of 7-deaza-2'-deoxy-
guanosine and its incorporation into oligo and poly-
nucleotides for the purpose of providing information on
base pairing and enzyme recognition is in progress.

55 Seela and Kehne, Tetrahedron, 41(22), 5387-5392
(1985) disclose the preparation of 2'-deoxy-
tubercidylyl(3'→5')-2'-deoxytubercidin, i.e. 7-deaza-2'-
deoxyadenosinyl(3'→5')-7-deaza-2'-deoxyadenosine, for
study of its hypochromicity and its stability to nuclease
60 cleavage, by condensation of 3'-O-[(N,N-diisopropylamino)-
methoxyphosphanyl]-5'-O-(4,4'-dimethoxytrityl)-7-deaza-6-
benzoyl-2'-deoxyadenosine with 3'-O-(t-butylmethoxysilyl)-7-
deaza-2'-deoxyadenosine and removal of the O-methyl, O-
dimethoxytrityl and N-benzoyl protecting groups.

65 Winkeler and Seela, Liebigs Ann. Chem., 708-721
(1984) disclose 7-deaza-7-methyl-2'-deoxyguanosine and its
synthesis from 2,4-diamino-6-hydroxypyrimidine.

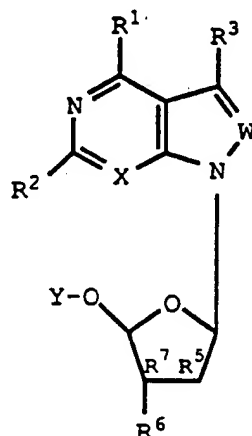
Seela and Kehne, Liebigs Ann. Chem., 876-884
(1983) disclose the preparation of 7-deaza-2'-deoxy-
70 adenosine.

Seela and Driller, Nucl. Acid. Res., 14, 2319-
2332 (1986) disclose the preparation, by solid phase
synthesis via the nucleoside phosphoramidites, of
octadecanucleotides containing the EcoRI endonuclease
75 recognition site and 7-deaza-2'-deoxyguanine and kinetic
studies on their cleavage by EcoRI.

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Seela, Tran-Thi and Franzen, Biochem., 21, 4338-4343 (1982) disclose the preparation of polymers of 7-deazaguanosine for study of their hypochromicity, melting profiles and circular dichroism spectra.

EPO Application 286,028, published October 12, 1988, discloses 7-deazapurine nucleosides of the formula:



where:

- 85 X is N or a =CH group;
W is N or a =CR⁴ group;
R¹, R², R³ and R⁴ are the same or different hydrogen, halogen, lower-alkyl, hydroxy, mercapto, lower-alkylthio, lower-alkoxy, arylalkyl, arylalkoxy, aryloxy or a mono or di-substituted amino group;
90 R⁵ is hydrogen or hydroxy;
R⁶ and R⁷ are hydrogen or one or both can be halogen, cyano, azido or a mono or di-substituted amino group, and wherein one of R⁶ and R⁷ can be hydroxy when X is a =CH group and furthermore R⁵ and R⁷ together can be a second bond between the C₂' and C₃' positions and Y is hydrogen or a mono, di or triphosphate.

95 The compounds are stated to be useful in nucleic acid sequencing, and as antiviral agents

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BACKGROUND OF THE INVENTION

An antisense compound is a compound that binds
105 to or hybridizes with a nucleotide sequence in a RNA or DNA
to inhibit the function or synthesis of the nucleic acid.
Because of their ability to hybridize with both RNA and
DNA, antisense compounds can interfere with gene expression
at the level of transcription, RNA processing or
110 translation.

Antisense molecules can be designed and
synthesized to prevent the transcription of specific genes
to mRNA by hybridizing with genomic DNA and directly or
indirectly inhibiting the action of RNA polymerase. A
115 theoretical advantage of targeting DNA is that only small
amounts of antisense compounds may be needed to achieve a
therapeutic effect. Alternatively, antisense compounds can
be designed and synthesized to hybridize with RNA to
inhibit post-transcriptional modification (RNA processing)
120 or protein synthesis (translation) mechanisms or to affect
mRNA stability. Exemplary target RNAs are messenger RNA
(mRNA), transfer RNA (tRNA), ribosomal RNA (rRNA)
heterogenous nuclear RNA (hnRNA) and the like. Examples of
processing and translation mechanisms include splicing of
125 pre-mRNA to remove introns, capping of the 5' terminus of
mRNA, transport to the cytoplasm, hybridization arrest and
ribonuclease H mediated mRNA hydrolysis.

At the present time, however, the development of
practical scientific and therapeutic applications of anti-
130 sense technologies is hampered by a number of technical
problems. Synthetic antisense molecules are susceptible to
rapid degradation by nucleases that exist in target cells.
The oligonucleotide sequences of antisense DNA or RNA, for
example, are destroyed by exonucleases acting at either the
135 5' or 3' terminus of the nucleic acid. In addition,
endonucleases can cleave the DNA or RNA at internal
phosphodiester linkages between individual nucleotides. As
a result of such cleavage, the effective half-life of
administered antisense compounds is very short,

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140 necessitating the use of large, frequently administered,
dosages.

Another problem is the extremely high cost of producing antisense DNA or RNA using available semiautomatic DNA synthesizers.

145 A further problem relates to the delivery of antisense agents to desired targets within the body and cell. Antisense agents targeted to genomic DNA must gain access to the nucleus (i.e. the agents must permeate the plasma and the nuclear membrane). The need for increased
150 membrane permeability (increased hydrophobicity) must be balanced, however, against the need for aqueous solubility (increased hydrophilicity) in body fluid compartments such as the plasma and cell cytosol.

A still further problem relates to the stability
155 of antisense agents whether free within the body or hybridized to target nucleic acids. Oligonucleotide sequences such as antisense DNA are susceptible to steric reconfiguration around chiral phosphorus centers.

Gene targeting via antisense agents is the
160 predicted next step in human therapeutics [Armstrong, Business Week March 5, 1990, page 88]. The successful application of antisense technology to the treatment of disease, however, requires finding solutions to the problems set forth above.

165 One approach to preparing antisense compounds that are stable, nuclease resistant, inexpensive to produce and which can be delivered to and hybridize with nucleic acid targets throughout the body is to synthesize oligonucleotide sequences having incorporated therein
170 modified adenine or guanine purine bases which are capable of hybridizing with their complementary respective thymine or cytosine bases but which are less susceptible to attack by exo- or endonucleases and which thus stabilize the oligonucleotide sequences to enzymatic degradation This
175 invention is directed to such an approach.

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SUMMARY OF THE INVENTION

In a product aspect the invention relates to
180 oligonucleotides incorporating a sequence of the normal DNA
bases, adenine (A), thymine (T), guanine (G) and cytosine
(C) in the required sequences for hybridization with a
given DNA or RNA base sequence and in which one or more of
the normal bases are replaced by a modified 7-deazaadenine
185 or 7-deazaguanine.

In a method aspect, the invention relates to a
method of inhibiting nuclease degradation of
oligonucleotides comprising incorporating one or more
modified 7-deazaadenine or 7-deazaguanine nucleosides into
190 a normal DNA base sequence.

In a further method aspect, the invention
relates to a method of inhibiting gene expression in a
cellular system comprising introducing into a cellular
system a composition containing an oligonucleotide which
195 incorporates one or more modified 7-deazaadenine or 7-
deazaguanine nucleosides.

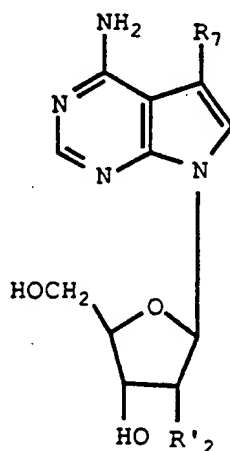
In a composition aspect, the invention relates
to compositions for inhibiting gene expression comprising
an oligonucleotide which incorporates one or more modified
200 7-deazaadenine or 7-deazaguanine nucleosides in a pharma-
ceutically acceptable carrier.

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DESCRIPTION OF THE PREFERRED EMBODIMENTS

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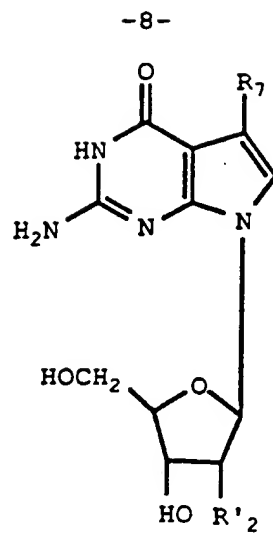
More specifically this invention relates to oligonucleotides incorporating a sequence of nucleotides of the normal DNA bases, i.e. adenine, thymine, guanine and cytosine, in the required sequence for hybridization with a given DNA or RNA base sequence and in which one or more of the normal bases are replaced by a 7-deazaadenine- β -D-ribofuranosyl or β -D-2'-deoxyribofuranosyl nucleoside of the formula:



Ia

215

or a 7-deazaguanine- β -D-ribofuranosyl- or β -D-2'-deoxyribofuranosyl nucleoside of the formula:



Ib

220 where

R'_2 is hydrogen or hydroxy; and

R_7 is hydrogen or lower-alkyl

with the provisos that the oligonucleotides incorporate (1)
no EcoRI endonuclease recognition sites, (2) no repeating
225 GC or CG sequences, and (3) no repeating AT or TA
sequences.

Preferred oligonucleotides within the ambit of
the invention are those which incorporate from one to four
of the 7-deazaadenine or 7-deazaguanine nucleosides of
230 formulas Ia or Ib in the polymer.

Other preferred oligonucleotides are those in
which the 7-deazaadenine or 7-deazaguanine nucleosides are
incorporated within the three nucleotide units at either or
both the 3' and the 5'-ends of the oligomer, which
235 oligomers are particularly stable to exonuclease
degradation.

Still other preferred oligonucleotides are those
in which the 7-deazaadenine or 7-deazaguanine nucleosides
are incorporated internally in the oligonucleotide
240 sequence, which oligonucleotides are particularly stable to
endonuclease degradation.

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Particularly preferred oligomers are those which incorporate nucleotides derived from the nucleosides of formula Ia wherein:

245 R'₂ and R₇ are hydrogen, i.e. 7-deaza-2'-deoxy-adenosine (9-β-D-2'-deoxyribofuranosyl-7-deazaadenine), hereinafter identified as nucleotide W;

 R'₂ is hydroxy and R₇ is hydrogen, i.e. 7-deaza-adenosine (9-β-D-ribofuranosyl-7-deazaadenine), hereinafter identified as nucleotide W';

250 R'₂ is hydrogen and R₇ is lower-alkyl, i.e. 7-deaza-2'-deoxy-7-methyladenosine (9-β-D-2'-deoxyribofuranosyl-7-deaza-7-methyladenine), hereinafter identified as nucleotide X; and

255 R'₂ is hydroxy and R₇ is lower-alkyl, i.e. 7-deaza-7-methyladenosine (9-β-D-ribofuranosyl-7-deaza-7-methyladenine), hereinafter identified as nucleotide X';

and nucleosides of formula Ib wherein:

260 R'₂ and R₇ are hydrogen, i.e. 7-deaza-2'-deoxy-guanosine (9-β-D-2'-deoxyribofuranosyl-7-deazaguanine), hereinafter identified as nucleotide Y;

 R'₂ is hydroxy and R₇ is hydrogen, i.e. 7-deaza-guanosine (9-β-D-ribofuranosyl-7-deazaguanine), hereinafter identified as nucleotide Y';

265 R'₂ is hydrogen and R₇ is lower-alkyl, i.e. 7-deaza-2'-deoxy-7-methylguanosine (9-β-D-2'-deoxyribofuranosyl-7-deaza-7-methylguanine), hereinafter identified as nucleotide Z; and

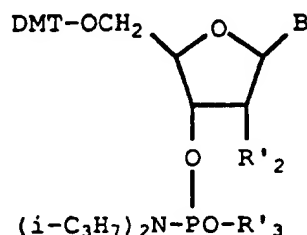
270 R₂ is hydroxy and R₇ is lower-alkyl, i.e. 7-deaza-7-methylguanosine (9-β-D-ribofuranosyl-7-deaza-7-methylguanine), hereinafter identified as nucleotide Z'.

As used herein the term lower-alkyl means a
275 saturated, aliphatic, straight or branched chain hydrocarbon radical containing from one to four carbon atoms and thus includes methyl, ethyl, propyl, isopropyl and butyl.

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The oligomers useful in the practice of the invention comprise sequences of from about 6 to 200 bases, preferably from about 12 to about 24 bases, and most preferably 15 bases in which one or more of the nucleosides are replaced by the modified 7-deazaadenine and 7-deazaguanine nucleosides of formulas Ia or Ib.

The oligonucleotides of the invention are prepared by solid phase synthesis according to well known procedures [Sinha et al., Nucl. Acid Res., 12, 4539-4557 (1984)] from protected 9-[3'-O-[(N,N-diisopropylamino) (R'₃-oxy)phosphanyl]-5'-O-(4,4'-dimethoxytrityl)purine nucleosides of the formula:



where

B represents the bases adenine (A), thymine (T), cytosine (C), guanine (G) or the modified 7-deazaadenine or 7-deazaguanine bases corresponding to the bases in the nucleosides of formulas Ia and Ib;

DMT represents the dimethoxytrityl group (i.e., the 4,4'-dimethoxytriphenylmethyl group); and

R'₂ has the meanings given above and where the 2-amino or 6-amino group of the respective guanine/7-deazaguanine, adenine/7-deazaadenine moieties are protected with a protecting group such as a benzoyl or isobutyryl group.

305

Particularly preferred solid phase syntheses are those such as described by Matteucci and Caruthers, J. Am. Chem. Soc.,

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103, 3185-3191 (1981) and Gait, *Oligonucleotide Synthesis: A Practical Approach*, Ed. by M.J. Gait, 35-81, IRL Press, Washington, D.C. 1984.

The initial step in solid phase synthesis is attachment of a nucleoside to a solid support, preferably a controlled pore glass (CPG) support. The nucleoside is preferably attached to the CPG via a succinate linkage at the 3'-hydroxy position of the nucleoside. Other means of attaching nucleosides to solid supports are known and readily apparent to those skilled in the oligonucleotide synthesis art.

Following attachment of the first nucleoside to the solid support, chain elongation occurs via the sequential steps of removing the 5'-hydroxy protecting group, activating the 5'-hydroxy group in the presence of a phosphoramidite reagent, adding the desired nucleoside, capping the unreacted nucleoside and oxidizing the phosphorus linkage. The protecting group, preferably DMT, at the 5'-hydroxy position of the attached nucleoside is removed with acid, preferably trichloroacetic acid.

Activating reagents that can be used in accordance with this method are well known to those skilled in the art. Preferred activating reagents are tetrazole and activator gold (Beckman Instr. Inc., Palo Alto, CA).

The activation step occurs in the presence of the added nucleoside and a trityldiolcyanophosphine compound, which compound replaces the nucleoside phosphoramidite of conventional synthetic methods. Unreacted chains are terminated or capped with capping reagents such as acetic anhydride and N-methylimidazole.

The labile trivalent phosphorus linkage is oxidized, preferably with iodine, to the stable, pentavalent phosphodiester linkage of the oligonucleotide.

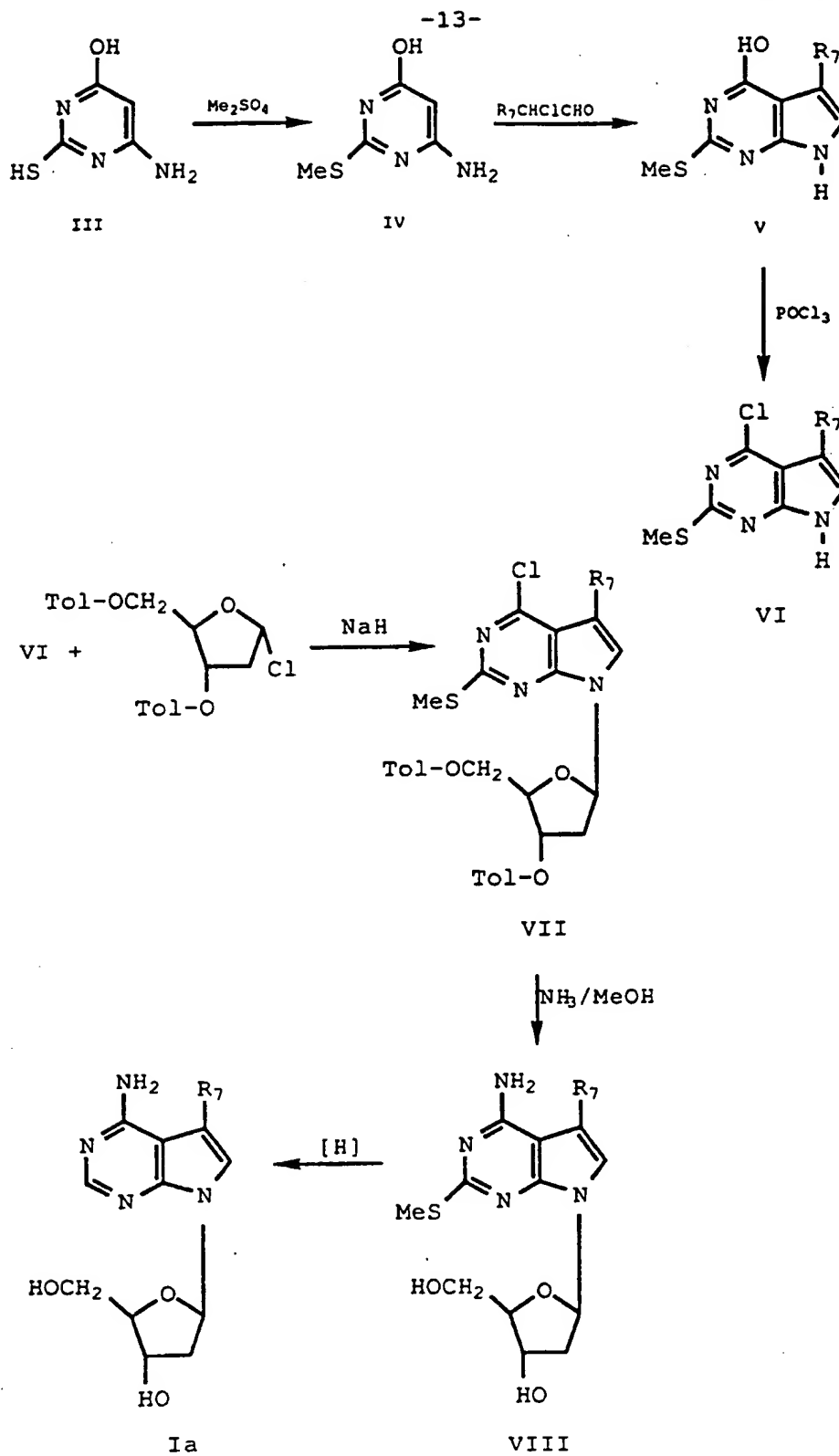
After the desired oligonucleotide chain assembly is complete, the phosphate protecting groups are removed, the chains are separated from the solid support and the base protecting groups are removed by conventional methods. (Gaits, *supra* at 67-70.)

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The compounds of the present invention are useful in treating mammals with hereditary disorders or diseases associated with altered genetic expression mechanisms. Examples of such diseases are viral infections
350 such as HIV, cytomegalovirus, herpes simplex, hepatitis B, papilloma virus and picornavirus; cancers of the lung, colon, cervix, breast and ovary; inflammatory diseases; and diseases of the immune system such as acquired immunodeficiency syndrome (AIDS), hematological neoplasma
355 and hyperproliferative disorders. [Armstrong, supra at 89; Klausner, Biotechnology, 8, 303, 304 (1990).]

The protected purine nucleosides of formula II required for the preparation of the oligomers of the invention can be prepared by the methods described by Seela
360 and Kehne (1987) supra; Seela and Kehne, Tetrahedron 41(22), 5387-5892 (1985); Seela and Driller (1989) supra; and Seela and Driller (1985) supra which involve benzylation of unprotected amino groups in the unprotected nucleosides of formulas Ia and Ib, or the unprotected 7-
365 deazapurine nucleosides of adenine, guanine or cytosine, formation of the 5'-dimethoxytrityl ether and converting the product to the 3-phosphoramidite.

The corresponding unprotected 7-deazapurine nucleosides of formula Ia and formula Ib can be prepared by
370 the methods described by Seela and Kehne (1987) supra, Seela and Driller (1989) supra, Tran-Thi supra, Seela and Driller (1985) supra, Winkeler and Seela (1983) supra, Winkeler and Seela (1984) supra and Seela and Kehne (1983) supra. 7-Deaza-2'-deoxy-7-lower-alkyladenines (formula Ia,
375 R₂=H) can be prepared by the following reaction scheme.



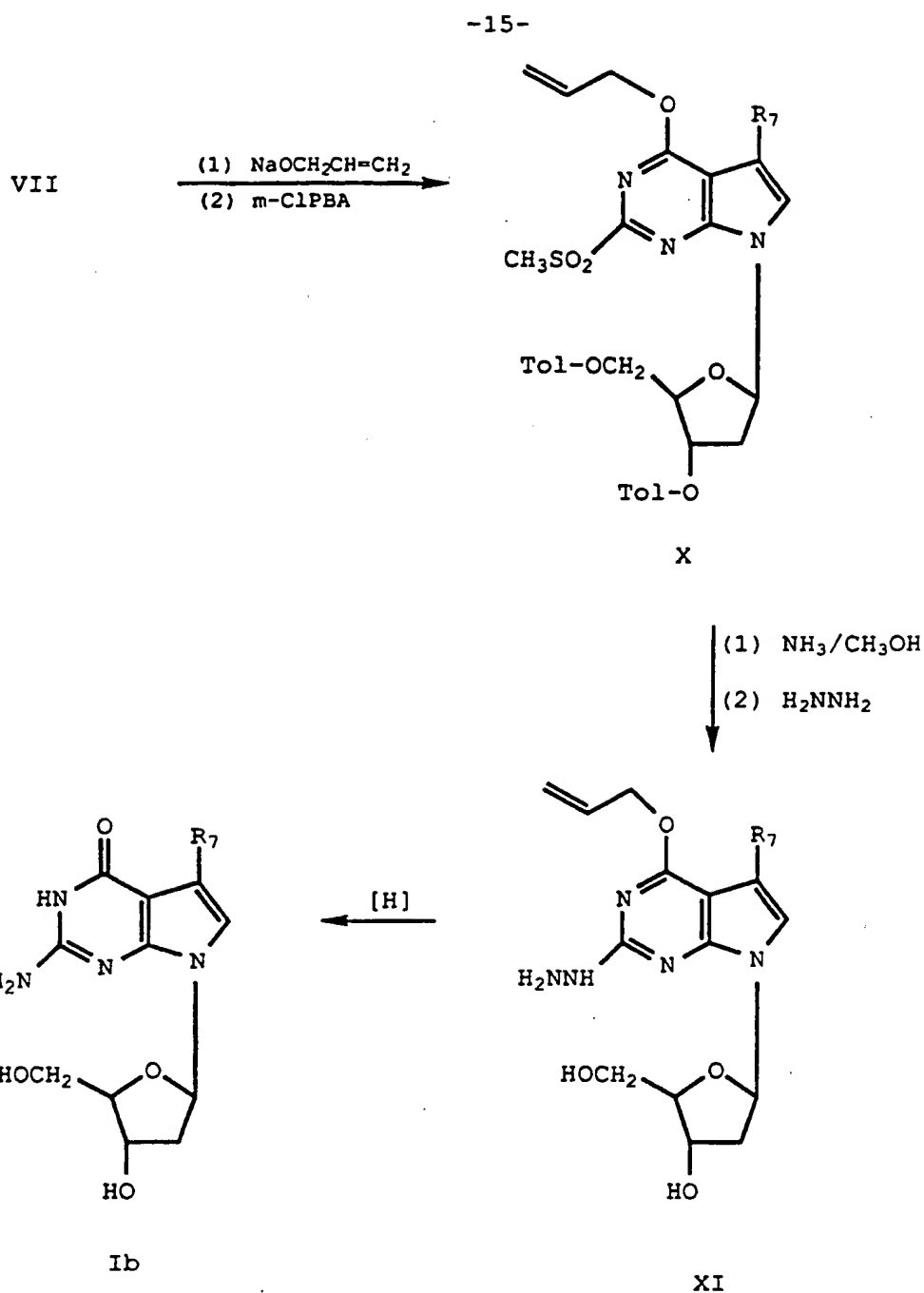
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where R₇ is lower-alkyl, and Tol is the p-toluoyl group.

Thus 6-amino-2-thiouracil (III) is treated with an alkylating agent, for example dimethyl sulfate, methyl bromide or methyl iodide, in the presence of a base, for example an alkali metal carbonate, in an organic solvent such as dimethylformamide (hereinafter DMF) or acetone. Using the procedure described by Winkeler and Seela (1984) supra, the resulting 2-methylthio-4-amino-6-hydroxypyrimidine (IV) is converted to the compound of formula VII by reaction of the compound of formula IV with an appropriate 2-chloro-lower-alkanal in the presence of an alkali metal carbonate and a tetra-lower-alkyl ammonium halide, conversion of the resulting 4-hydroxy-2-methylthio-5-lower-alkyl-7H-pyrrolo[2,3-d]pyrimidine of formula V to the corresponding 4-chloro-2-methylthio-5-lower-alkyl-7H-pyrrolo[2,3-d]pyrimidine of formula VI which is reacted with 1-chloro-2-deoxy-3,5-di-O-(p-toluoyl)- α -D-erythro-pentofuranose in the presence of a base to give a 4-chloro-2-methylthio-5-lower-alkyl-7-[2'-deoxy-3',5'-di-O-(p-toluoyl)- β -D-erythro-pentofuranosyl]-7H-pyrrolo[2,3-d]pyrimidine of formula VII. Thereafter the compound of formula VII, in an appropriate organic solvent inert under the conditions of the reaction such as a lower-alkanol, is heated with ammonia in an autoclave to give the 4-amino-2-methylthio-5-lower-alkyl-7-[2'-deoxy- β -D-erythro-pentofuranosyl]-7H-pyrrolo[2,3-d]pyrimidine (alternatively named as 9- β -D-2'-deoxyribofuranosyl-2-methylthio-7-deaza-7-lower-alkyladenine) of formula VIII. Reductive dethiomethylation of the latter in an inert organic solvent, for example a lower alkanol, over Raney nickel affords the desired 9- β -D-2'-deoxyribofuranosyl-7-deaza-7-lower-alkyladenine of formula Ia wherein R₂' is hydrogen. The latter can then be converted to the protected 7-deaza-7-lower-alkyladenines of formula II as described before.

Likewise, 9- β -D-2'-deoxyribofuranosyl-7-deaza-7-lower-alkylguanine of formula Ib can be prepared by the following reaction scheme;

415



These can then be converted to the 9- β -D-2'-
deoxyribofuranosyl-7-deaza-7-lower-alkylguanines of formula
420 II, as described before.

The pharmaceutical compositions of the present
invention include one or more of the compounds of this

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invention formulated into compositions together with one or more non-toxic physiologically acceptable carriers, 425 adjuvants or vehicles which are collectively referred to herein as carriers, for parenteral injection, for oral administration in solid or liquid form, for rectal or topical administration, and the like.

The compositions can be administered to humans 430 and animals either orally, rectally, parenterally (intravenously, intramuscularly or subcutaneously), intracisternally, intravaginally, intraperitoneally, locally (powders, ointments or drops), or as a buccal or nasal spray.

435 Compositions suitable for parenteral injection may comprise physiologically acceptable sterile aqueous or nonaqueous solutions, dispersions, suspensions or emulsions and sterile powders for reconstitution into sterile injectable solutions or dispersions. Examples of suitable 440 aqueous and nonaqueous carriers, diluents, solvents or vehicles include water, ethanol, polyols (propylene glycol, polyethyleneglycol, glycerol, and the like), suitable mixtures thereof, vegetable oils (such as olive oil) and injectable organic esters such as ethyl oleate. Proper 445 fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersions and by the use of surfactants.

These compositions may also contain adjuvants 450 such as preserving, wetting, emulsifying, and dispensing agents. Prevention of the action of microorganisms can be ensured by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, and the like. It may also be desirable to include isotonic 455 agents, for example sugars, sodium chloride and the like. Prolonged absorption of the injectable pharmaceutical form can be brought about by the use of agents delaying absorption, for example, aluminum monostearate and gelatin.

If desired, and for more effective distribution, 460 the compounds can be incorporated into slow release or

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targeted delivery systems such as polymer matrices, liposomes, and microspheres. They may be sterilized, for example, by filtration through a bacteria-retaining filter, or by incorporating sterilizing agents in the form of
465 sterile solid compositions which can be dissolved in sterile water, or some other sterile injectable medium immediately before use.

Solid dosage forms for oral administration include capsules, tablets, pills, powders and granules. In
470 such solid dosage forms, the active compound is admixed with at least one inert customary excipient (or carrier) such as sodium citrate or dicalcium phosphate or (a) fillers or extenders, as for example, starches, lactose, sucrose, glucose, mannitol and silicic acid, (b) binders,
475 as for example, carboxymethylcellulose, alginates, gelatin, polyvinylpyrrolidone, sucrose and acacia, (c) humectants, as for example, glycerol, (d) disintegrating agents, as for example, agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain complex silicates and sodium
480 carbonate, (e) solution retarders, as for example, paraffin, (f) absorption accelerators, as for example, quaternary ammonium compounds, (g) wetting agents, as for example, cetyl alcohol and glycerol monostearate, (h) adsorbents, as for example, kaolin and bentonite, and (i)
485 lubricants, as for example, talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate or mixtures thereof. In the case of capsules, tablets and pills, the dosage forms may also comprise buffering agents.

490 The molecular structures of the compounds were established on the basis of a study of the nmr, infrared and mass spectra, and their purities were established by HPLC and chemical analysis for their elements.

Nuclease Stability

495 Antisense oligonucleotides modified in accordance with the present invention were evaluated (and compared against unmodified oligonucleotides) for their stability in the presence of 10% (v/v) fetal bovine serum

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(FBS) in RPMI 1640 cell culture media containing 20 mM
500 HEPES buffer (complete media). FBS and human serum are
known to exhibit 3'→5' exonuclease activity. This
represents the only nuclease activity we have been able to
detect in FBS, human serum and human plasma.
Oligonucleotide samples were incubated at 37°C in complete
505 media over a six hour time period and the amount of parent
compound determined using an HPLC-based procedure.

Measurement of DNA/DNA Duplex Melting Temperatures

Oligonucleotide concentrations were determined
spectrophotometrically utilizing extinction coefficients at
510 260 nm calculated using the method and values presented by
Warsaw, Cantor, and Tinoco [CRC Handbook of Biochemistry
and Molecular Biology (G.D. Fasman, editor) 1:589 (1975)].
Equimolar concentrations of oligonucleotide and its comple-
mentary sequence were combined (in 0.1 mM EDTA, 10 mM
515 sodium phosphate, 0.1 M NaCl, pH 7.0) heated to 80°C and
allowed to cool slowly at room temperature. Samples were
allowed to remain at room temperature for about 2.5 hours.
Samples were then heated at a rate of 0.5°C/min (25°C-75°C)
in a thermostatically controlled heat block and absorbance
520 monitored at 260 nm using a Perkin Elmer Lambda 4C UV
spectrophotometer. A₂₆₀ measurements were taken every 15
seconds. Data were transferred to a DEC VAX for data
analysis using RS/1 data analysis software. T_m's were
determined from a plot of dA₂₆₀/dT vs. temperature. The T_m
525 is that temperature at which dA₂₆₀/dT is maximum.

Inhibition of Rabbit Alpha Globin mRNA Translation

Cell free translation of rabbit globin mRNA
(Bethesda Res. Labs, Gaithersburg, MD) +/- 6.5 units/5 uL
E.coli RNase H (Boehringer Mannheim, Indianapolis, IN) with
530 the addition of antisense oligonucleotides was carried out
using rabbit reticulocyte lysate (Promega, Madison, WI) in
a total volume of 50 uL. 25 uCi of 35S-methionine (New
England Nuclear, Boston, MA) was added to each translation
reaction. Translations were incubated at 30°C for 10 min.,
535 after which time samples were snap frozen on dry ice.
Alpha and beta globin chains were separated using SDS-

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polyacrylamide gel electrophoresis. The 15 cm gels were prepared using electrophoresis buffer (0.1 M sodium phosphate, pH 7.2 containing 1.0 g of SDS/L) and contained
540 12.5% acrylamide and 0.6% bisacrylamide. Aliquots (1 uL) of translation reactions were diluted with 11 uL of loading buffer consisting of electrophoresis buffer, 1.1% 2-mercaptoethanol, 2.5% glycerol and bromphenol blue. Samples were denatured by heating to 100°C for 3 min.
545 before loading onto gels. The gels were run for 18 hours at 30 mAMP. After electrophoresis, gels were stained with coomassie blue, dried and autoradiographed at -70°C for 16 hours.

Quantitation of the effects of alpha globin
550 directed antisense oligonucleotides on the synthesis of alpha globin was done by scanning the autoradiographs using an Ultrascan XL laser densitometer (LKB/Bromma) linked to an AT&T PC6300 computer. Data were collected, displayed and integrated with the Gelscan XL data analysis software
555 package (LKB/Bromma). Effects of oligomers on protein synthesis were expressed as a percent of control alpha globin synthesis.

The following examples will further illustrate the invention without limiting it thereto. It will be
560 apparent to those skilled in the art that the embodiments disclosed may be readily modified by standard procedures to produce oligonucleotides of other lengths and with other sequences. Targets for synthesis will usually be chosen by substituting a 7-deazaadenine or 7-deazaguanine nucleoside
565 of formulas Ia or Ib in the sequence which is to be protected from nuclease degradation or which is complementary to a sequence which is to be blocked.

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Preparation of the Protected NucleosidesPreparation 1

570 A stirred suspension of 0.8 g (20 mmole) of a
60% sodium hydride in hexane dispersion was decanted and
taken to dryness, resuspended in 100 ml of dry acetonitrile
and the suspension treated with 3.21 g (15 mmole) of 4-
575 chloro-5-methyl-2-methylthiopyrrolo[2,3-d]pyrimidine [Kondo
et al., Agric. Biol. Chem. 4(8), 1501-1507 (1977)]. The
mixture was stirred under nitrogen at room temperature for
one hour and then treated with 5.9 g (15 mmole) of 1-
chloro-2-deoxy-3,5-di-O-(p-toluoyl)- α -D-erythro-
pentofuranose added in portions. An additional 40 ml of
580 acetonitrile was added, the mixture stirred at 50°C for
about three and one half hours and then filtered and the
solid washed with acetonitrile and dried to give 6.1 g
(72%) of 4-chloro-5-methyl-2-methylthio-7-[α -D-erythro-
pentofuranosyllpyrrolo[2,3-d]pyrimidine, m.p. 163-163.5°C.

585 The latter (11.0 g, 19.4 mmole) was suspended in
225 ml of a solution of absolute methanol saturated with
ammonia and the mixture heated in an autoclave for nineteen
hours at 125°C with stirring. The reaction mixture was
cooled in an ice/water bath, taken to dryness in vacuo, the
590 residue triturated sequentially with diethyl ether,
chloroform and acetone and the solid collected and dried to
give 2.65 g (45%) of 4-amino-5-methyl-2-methylthio-7-[α -D-
erythro-pentofuranosyllpyrrolo[2,3-d]pyrimidine, m.p. 187-
189°C.

595 The product (0.47 g, 1.5 mmole) suspended in 45
ml of n-propanol was treated with 3.1 g of wet Raney nickel
and the mixture heated under reflux for seven and a half
hours, then cooled and filtered. The filtrate was taken to
dryness and the residue recrystallized from water to give
600 0.26 g (72%) of 7-deaza-2'-deoxy-7-methyladenosine, m.p.
213.5-214.5°C.

A suspension of 0.22 g (0.83 mmole) of the
product in 8.5 ml of dry pyridine was cooled in an
ice/methanol bath and then treated dropwise with 0.5 ml
605 (approximately 5 equivalents) of trimethylchlorosilane over

-21-

a few minutes. The mixture was treated with about 0.5 ml of benzoyl chloride, stirred under nitrogen at room temperature for about two hours, cooled in an ice bath again, treated with 1.65 ml of water and 1.7 ml of concentrated ammonium hydroxide, stirred under nitrogen at ambient temperature for about a half hour and then taken to dryness. The crude product was triturated with water followed by cyclohexane to give 0.4 g of 6-dibenzoyl-7-deaza-2'-deoxy-7-methyladenosine, 6.5 g (11.35 mmole) of which was hydrolyzed to the mono 6-benzoyl-7-deaza-2'-deoxy-7-methyladenosine by treatment with 200 ml of a 50% solution of 1N sodium hydroxide in ethanol and then acidifying with 2N hydrochloric acid. There was thus obtained 3.61 g (86%) of product, m.p. 172-175°C.

The latter (1.75 g, 4.75 mmole), in about 50 ml of dry pyridine, was treated with 1.86 g (5.23 mmole) of 4,4'-dimethoxytrityl chloride and the mixture stirred at ambient temperature under nitrogen for about four hours and 17 mL of methanol added then taken to dryness in vacuo. The product was purified by chromatography on silica gel, eluting the product with 3% methanol in chloroform. There was thus obtained 1.97 g (62%) of 6-benzoyl-7-deaza-2'-deoxy-7-methyl-5'-dimethoxytrityladenosine, m.p. 112-115°C.

A solution of 0.9 g (1.3 mmole) of the product in 7.5 ml of dry THF was treated with 1.0 ml (5.7 mmole) diisopropylamine and the solution treated dropwise with 1.0 ml (4.5 mmole) of chloro- β -cyanoethoxy-N,N-diisopropylaminophosphine over a period of about 40 minutes while stirring under nitrogen. The mixture was then stirred at ambient temperature under nitrogen for about 40 minutes and taken to dryness in vacuo to give the crude product which was purified by chromatography on silica gel, the product being eluted with helium saturated ethyl acetate. There was thus obtained 0.62 g (55%) of 6-benzoyl-7-deaza-2'-deoxy-7-methyl-3'-O-[(N,N-diisopropylamino)- β -cyanoethoxyphosphanyl]-5'-dimethoxytrityladenosine, m.p. 73-76°C.

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Preparation 2

Reaction of the 4-chloro-5-methyl-2-methylthio-
645 7-(α -D-erythro-pentofuranosyl)pyrrolo[2,3-d]pyrimidine
described in Preparation 1 above with sodium 2-
propenyloxy in DMF affords 5-methyl-2-methylthio-4-(2-
propenyloxy)-7-(α -D-erythro-pentofuranosyl)pyrrolo[2,3-
d]pyrimidine, which, on oxidation with two molar
650 equivalents of 3-chloroperbenzoic acid in methylene
chloride, affords 5-methyl-2-methylsulfonyl-4-(2-
propenyloxy)-7-(α -D-erythro-pentofuranosyl)pyrrolo[2,3-d]-
pyrimidine. Reaction of the product with hydrazine
affords 5-methyl-2-hydrazino-4-(2-propenyloxy)-7-(α -D-
655 erythro-pentofuranosyl)pyrrolo[2,3-d]pyrimidine. Reduction
of the product with for example Raney nickel affords 7-
deaza-2'-deoxy-7-methylguanosine.

Proceeding in a manner similar to that described
in Preparation 1 above, the latter is treated sequentially
660 first with trimethylchlorosilane in the presence of
pyridine, then with isobutyric anhydride and then with
concentrated ammonium hydroxide to give 2-isobutyryl-7-
deaza-2'-deoxy-7-methylguanosine, which, on reaction with
one molar equivalent of trityl chloride in the presence of
665 dry pyridine, affords 2-isobutyryl-7-deaza-2'-deoxy-7-
methyl-5'-tritylguanosine. Reaction of the latter with one
molar equivalent of chloro- β -cyanoethoxy-N,N-
diisopropylaminophosphine affords 2-isobutyryl-7-deaza-2'-
deoxy-7-methyl-3'-O-[(N,N-diisopropylamino)- β -
670 cyanoethoxyphosphanyl]-5'-tritylguanosine.

Preparation of the OligomersExamples 1-8

The oligomers of Examples 1-8 below, as well as
675 the control samples, were synthesized using standard
procedures on an Applied Biosystems model 380B DNA
synthesizer to provide the DNA oligomers described below.
In all successful couplings a 10-fold excess of the
appropriate protected nucleoside monomer was used. In the

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680 examples, the letters A, G, W, X, C and T have the
following nucleic acid base meanings:

685 A: adenine
 G: guanine
 W: 7-deazaadenine
 X: 7-methyl-7-deazaadenine
 C: cytosine
690 T: thymine

Table 1

	<u>Example</u>	<u>Structures (5'→3')</u>
695	Control	AAA AAA AAA AAA AAA
	Control	TTT TTT TTT TTT TTT
	C-MYC-Sense	ATG CCC CTC AAC GTT
	Antisense	AAC GTT GAG GGG CAT
700	1	AAA AAA AAA AAA AWA
	2	AAA AAA AAA AAA WWA
	3	AAA AAA AAA AAA AXA
	4	AAA AAA AAA AAA XXA
	5	AAA AAA AAA AAA XAA
705	6	XXC GTT GXG GGG CXT
	7	CCT TCT CXG TCG GXT
	8	WWC GTT GWG GGG CWT

710 The melting temperatures obtained for each of
the oligomers described above are given in Table 2 below.

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Table 2

715

<u>Example</u>	<u>C (μm)</u>	<u>T_m (°C)</u>	<u>Δ T_m</u>
Control (Antisense)	7.5	42.2	
Control (Antisense)	8.8 ^a	65.1 ^a	
1	7.5	41.0	-1.2
2	7.5	39.4	-2.8
3	7.5	42.2	0.0
4	7.5	41.4	-0.8
5	7.5	41.6	-0.6
6		64.5	-0.6
7			
8	10 ^a	63.4	-1.7

(a) Avg. of two values

720 The stabilities of the oligomers of the invention to 3-exonuclease, in comparison with controls, are given in Table 3 below.

Table 3

725

<u>Example</u>	<u>1/2 Life (Minutes)</u>	<u>Ratio</u>
Control (Antisense)	70	
Control (Antisense)	70	
1	265	3.8
2	205	2.9
3	185	2.6
4	155	2.2
5	120	1.7
6	175	2.5
7	115 ^a	7.2
8	20	0.3

-25-

- (a) The corresponding unmodified oligomer, CCT TCT CAG TCG GAC had a half life of 16 minutes.

730

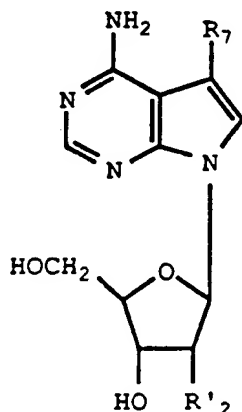
The oligomer of Example 6 was found to inhibit translation to $13 \pm 4\%$ of control in the absence of RNase H and $5 \pm 1\%$ in the presence of RNase H in comparison with corresponding unmodified oligomer which inhibited

735 translation to $21 \pm 4\%$ of control in the absence of RNase H and 14% in the presence of RNase H.

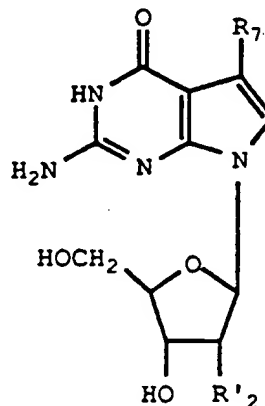
-26-

We claim:

1. An oligonucleotide sequence of from 6 to 200
 740 nucleotides containing one or more nucleotides derived from
 the 7-deazaadenine or 7-deaza-7-lower-alkyladenine
 nucleosides of formula Ia or the 7-deazaguanine or 7-deaza-
 7-lower-alkylguanine nucleosides of formula Ib:



Ia



Ib

745

where:

R'2 is hydrogen or hydroxy; and

R7 is hydrogen or lower-alkyl,

- with the provisos that the oligonucleotides incorporate (1)
 750 no EcoRI endonuclease recognition site and (2) no repeating
 GC or CG sequences.

2. An oligonucleotide according to Claim 1 wherein
 in the nucleotides derived from the nucleosides of formula
 Ia:

755

R'2 and R7 are hydrogen;

R'2 is hydroxy and R7 is hydrogen;

R'2 is hydrogen and R7 is lower-alkyl; or

R'2 is hydroxy and R7 is lower-alkyl

- and wherein in the nucleotides derived from the nucleosides
 760 of formula Ib:

R'2 and R7 are hydrogen;

R'2 is hydroxy and R7 is hydrogen;

R'2 is hydroxy and R7 is lower-alkyl; or

R'2 is hydrogen and R7 is lower-alkyl.

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- 765 3. An oligonucleotide according to Claim 2 wherein
R'₂ is hydrogen.
4. An oligonucleotide according to Claim 2 wherein
R'₂ is hydroxy.
5. An oligonucleotide according to Claim 3
770 containing from 12 to 24 bases.
6. An oligonucleotide according to Claim 5
containing 15 bases.
7. An oligonucleotide according to Claim 4
containing from 12 to 24 bases.
- 775 8. An oligonucleotide according to Claim 7
containing 15 bases.
9. An oligonucleotide according to Claim 6 wherein
the modified purine nucleosides are incorporated within the
three nucleotide units at either or both the 3'- and the
780 5'-ends of the oligomer.
10. An oligonucleotide according to Claim 8 wherein
the modified purine nucleosides are incorporated within the
three nucleotide units at either or both the 3'- and the
5'-ends of the oligomer.
- 785 11. An oligonucleotide according to Claim 6 wherein
the modified purine nucleosides are incorporated within the
three nucleotide units at either or both the 3'- and the
5'-ends or internally in the nucleotide sequence of the
oligomer.
- 790 12. An oligonucleotide according to Claim 8 wherein
the modified purine nucleosides are incorporated within the
three nucleotide units at either or both the 3'- and the
5'-ends or internally in the nucleotide sequence of the
oligomer.

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795 13. An oligonucleotide according to Claim 9 selected
from the group consisting of:

AAA AAA AAA AAA AWA
AAA AAA AAA AAA WWA
800 AAA AAA AAA AAA AXA
AAA AAA AAA AAA XXA
AAA AAA AAA AAA XAA

wherein A is adenine, W is 7-deazaadenine, X is 7-methyl-7-
deazaadenine.

805

14. An oligonucleotide according to Claim 11
selected from the group consisting of:

XXC GTT GXG GGG CXT
810 CCT TCT CXG TCG GXT
WWC GTT GWG GGG CWT

wherein;

G is guanine;
W is 7-deazaadenine;
815 X is 7-methyl-7-deazaadenine;
C is cytosine; and
T is thymine.

15. A method of inhibiting nuclease degradation of
820 an oligonucleotide which comprises incorporating within the
oligonucleotide a modified 7-deazaadenine or 7-deazaguanine
nucleoside according to Claim 1.

16. A method of inhibiting gene expression in a
cellular system which comprises introducing to the cellular
825 system an effective amount of an oligonucleotide according
to Claim 1.

17. A composition for inhibiting gene expression
comprising an oligonucleotide according to Claim 1 in a
pharmaceutically acceptable carrier.

INTERNATIONAL SEARCH REPORT

Int. Application No

PCT/US 94/02996

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C07H21/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	NUCLEIC ACIDS RESEARCH. vol. 12, no. 23, 11 December 1984, ARLINGTON, VIRGINIA US pages 8939 - 8949 A.ONO ET AL. 'Synthesis of Deoxyoligonucleotides Containing 7-deazaadenine: Recognition and Cleavage by Restriction Endonuclease Bgl II and Sau 3AI (Nucleosides and Nucleotides Part 55).' see the whole document --- -/--	1,2,4, 15-17

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

28 June 1994

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Scott, J

INTERNATIONAL SEARCH REPORT

Int. Application No
PCT/US 94/02996

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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INTERNATIONAL SEARCH REPORT

Int. Appl. No.
PCT/US 94/02996

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 94/02996

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 94/02996

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